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$\alpha_v\beta_6$ integrin expression in diseased and transplanted kidneys

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Background. Integrins have been implicated in the pathogenesis of a diverse range of kidney diseases. Herein, we provide the first detailed description of an epithelial restricted integrin, $\alpha_v\beta_6$, in kidney biopsies from patients suffering acute and chronic renal diseases and after transplantation.

Methods. Immunoperoxidase staining for β_6 was performed on 267 selected biopsy specimens from native ($N = 126$) and transplanted kidneys ($N = 141$) and scored semiquantitatively. The site of β_6 expression in tubules was determined using haematoxylin counterstaining and by colocalization with Tamm-Horsfall protein. Comparisons were made between subcategories of diseases of native kidneys and between "service" and "protocol" biopsies of transplanted kidneys.

Results. β_6, when present, is largely confined to the distal tubules and collecting ducts, colocated with Tamm-Horsfall protein. When sparsely present, it was often restricted to the tubular segment associated with the juxtaglomerular apparatus. It was found in tubular cells shed into the urine. β_6 was not expressed in thin membrane nephropathy, or in nonproliferative forms of glomerulonephritis, with the exception of focal and segmental glomerulosclerosis (FSGS). It was diffusely expressed where there was glomerular necrosis or thrombosis and in most forms of acute or chronic tubulointerstitial disease. β_6 was diffusely up-regulated in allografts biopsied for delayed function, in almost all kidneys that have clinical or subclinical rejection episodes and was prominent in chronic allograft nephropathy.

Conclusion. β_6 integrin is not normally expressed in adult native or transplanted kidneys but is commonly up-regulated in the distal tubule in disease. Our descriptive study suggests that it is a molecule worthy of further study.

Tissue repair processes are regulated, at least in part, by cell adhesion receptors and of all the families of cell adhesion molecules, the integrin family has been best characterized. Individual integrins comprise an α and β subunit in noncovalent association and there are now known to be 18 α subunits and 8 β subunits that can form at least 24 different heterodimers. These transmembrane receptors provide a structural and functional bridge between the extracellular matrix (ECM) and the intracellular cytoskeleton [1]. Cellular functions of integrins include induction of cell polarity and regulation of cell migration, proliferation, differentiation, and programmed cell death.

Multiple members of the α_v and β_1 integrin subfamilies have been implicated in various kidney diseases. For example, in IgA nephropathy β_1 integrins have been found to be localized to the mesangial cell membranes in close proximity to immune complex deposits [2]. α_2, α_3, and β_1 integrins have been identified on damaged tubules raising the possibility that altered β_1 integrin expression may play a role in tubulointerstitial scarring in this disease [3]. In acute renal failure as a consequence of ischemic injury it has been proposed that reorganization of β_1 integrins from basal to apical surfaces in injured tubular epithelium may facilitate epithelial detachment contributing to tubule obstruction and back flow of glomerular filtrate [4]. Moreover, $\alpha_3\beta_1$ at the podocyte basal plasma membrane is thought to play a role in the dysfunction of the glomerular wall characteristic of diabetic glomerulosclerosis [5].

Expression of α_v integrins in proximal and distal tubular epithelium has also been observed in glomerulo nephritis linked to the presence of macrophages within the interstitium and the presence of markers of disease progression (interstitial fibrosis and tubular atrophy) [6, 7]. The majority of kidney biopsies from patients with proteinuric glomerulonephritides, including membranous and focal sclerosing glomerulonephritis, have been shown to have up-regulated β_5 expression in tubular cells while the integrin is not expressed in nonproteinuric kidneys [8]. Among the α_v integrin subfamily a candidate integrin likely to play a major role in inflammatory diseases of the kidney is $\alpha_v\beta_6$. This epithelial-restricted integrin has been shown to bind fibronectin, tenascin, and vitronectin in a range of cell types [9–11]. $\alpha_v\beta_6$ is either not expressed or expressed at very low levels in normal epithelia; however, it becomes highly expressed during

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inflammation, wound healing, morphogenesis, and tumorigenesis [12, 13].

Breuss et al [12, 14] were the first to report $\alpha_v\beta_6$ integrin expression in the renal tubular epithelium of developing embryos, in diseased kidneys, and in rejected allografts. These investigators observed β_6 expression in distal tubules and collecting ducts in immunostained cryostat sections of native kidney tissue obtained from three patients suffering from hemolytic-uremic syndrome (HUS), arterial nephrosclerosis with tubular atrophy, and acute tubular necrosis, respectively [12]. In addition Breuss et al also analyzed cryostat sections prepared from rejected, nephrectomized renal transplants and in all cases up-regulation of β_6 expression was observed in distal tubules and ducts. Our longstanding interest in factors regulating β_6 expression prompted us to use an immunohistochemical staining technique applicable to preserved paraffin-embedded specimens. Herein, we provide the first detailed analysis of β_6 expression in biopsies from patients selected to have a comprehensive spectrum of native kidney diseases in various stages of evolution and also from patients with diverse problems related to renal transplantation.

METHODS

Tissues

Native kidney diseases. Human renal biopsy material taken for diagnostic purposes was selected retrospectively from stored specimens in our hospital's pathology service. Cases were selected to reflect a wide variety of native renal diseases with the histologic diagnosis having been established by conventional light microscopy, immunofluorescent staining, and electron microscopy. Of the 126 biopsies studied, seven were from patients with biopsies which were normal to light microscopy and the remainder were from various categories of kidney disease, namely glomerulonephritis (59 patients), tubulointerstitial disease (36), and miscellaneous conditions (thrombotic microangiopathy [10], diabetic glomerulosclerosis [12], and myeloma kidney [2]).

Renal transplants. In the last 5 years all our cadaveric and live donor transplant kidneys have undergone so-called "protocol" biopsies at time zero (on the operating table, postreperfusion) and at 3 months posttransplant. As part of an ongoing study these are stained for β_6 integrin expression, which is correlated with clinical, laboratory, and histologic outcomes. Biopsy findings in the first 50 such cases are presented here. As well, selected biopsies dating from as far back as 1992 were successfully stained to provide insight into the expression pattern of β_6 in various diagnostic categories of allograft dysfunction [i.e., delayed graft function without acute rejection (DGF) (nine patients), delayed graft function with acute rejection episode (DGF + ARE) (three patients), clinical acute rejection episodes (ARE) (10 patients), and chronic allograft nephropathy (CAN) (18 patients)].

Immunoperoxidase staining

All tissues were fixed in mercuric formalin, postfixed in Dubosq-Brazil fixative and processed to paraffin wax (graded alcohols, histolene, wax). Sections were cut at 3 μ, placed on silanized slides and oven dried at 60°C for 2 hours. Slides were deparaffinized and rehydrated followed by an iodine bath and aqueous sodium thiosulphate (hypo) solution to rid the sections of any mercury deposits. Antigen retrieval was performed by placing slides in 0.1% trypsin at 37°C for 20 minutes followed by washing with water before exposure of the sections to 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase. After washing with water again the slides were placed in Tris-buffered saline (TBS) for 10 minutes. An avidin-biotin blocking system (Dako, Botany, NSW, Australia) was used (10 minutes 1% avidin and 10 minutes 0.1% biotin with 10-minute TBS wash steps between) before applying normal goat serum (Newcastle Antisera, Newcastle, Australia) diluted 1:20 for 10 minutes. The normal goat serum was decanted before applying either the primary antibody of interest (mouse anti-integrin β_6 monoclonal antibody diluted 1:150 (Chemicon International, Temecula, CA, USA) or isotype-matched control mouse IgG1 (Auspep, Parkville, Australia) (used at a dilution of 1:150). The slides were incubated overnight in a moist chamber at 4°C and allowed to reach room temperature the following day before further washing in a TBS bath for 10 minutes. The secondary antibody, goat antimouse (Dako) (diluted 1:500) was then applied to the tissue sections for 30 minutes at room temperature. The slides were again washed in TBS for 10 minutes before application of peroxidase-labeled streptavidin (Dako) (diluted 1:800) for 30 minutes at room temperature followed by another 10-minute wash in TBS.

The chromogen diaminobenzidine (DAB) was prepared as per instructions (Dako) during the final 10-minute wash, applied to the slides, and the optimum staining time (usually 5 to 10 minutes) determined by microscopic examination. The stained slides were washed in water for 5 minutes and counterstained in Mayer's hematoxylin. The final preparation of the slides consisted of a series of graded alcohol dehydration steps and mounting with a coverslip using Ultramount Mountant (Fronine, Riverstone, NSW, Australia).

Staining for the presence of Tamm-Horsfall protein was performed using sheep antihuman Tamm-Horsfall polyclonal antibody (Chemicon) at a dilution of 1:40,000 followed by staining with secondary donkey antisheep antibody (Amersham, Amersham, UK) at a dilution of 1:500.

Staining using both β_6 integrin and Tamm-Horsfall anti sera was done by first staining for Tamm-Horsfall with

Table 1. Scoring system for $\beta_6$staining

Score	Description	% Tubules staining for β_6
Trace	Trace	0–5
1+	Focal	5–10
2+	Mild diffuse	10–20
3+	Moderate diffuse	20–30
4+	Very diffuse	30–40

A score of 4+ (30% to 40% of tubular cross-sections) means that virtually all distal tubules and collecting ducts (i.e., all nephrons) within the kidney expressed β_6.

DAB chromogen, and then staining for β_6 on a consecutive section.

The slides were assessed by means of light microscopy and photographed using a Nikon Coolpix 950 digital camera. The sections were scored for β_6 expression by two experienced renal histopathologists using a semiquantitative scoring system that is shown in Table 1.

Data analysis

This being a descriptive study β_6 scores were tabulated by disease categories to facilitate comparisons. The frequency distribution of β_6 scores was also represented graphically in dot-plots using mid-points of the β_6 score ranges to aid comparisons with linearity of scale. Post hoc statistical analysis of the categorical data was performed between some relevant disease categories using 2×2 contingency tables analyzed by two-tailed Fisher's exact test. *P* values obtained were used to highlight differences between two individual categories rather than to infer any clinical meaning to the data.

RESULTS

Patterns of β_6 integrin subunit expression in renal parenchyma

No β_6 staining was seen in biopsies that were normal to light microscopy yet had electron microscopic features to support a diagnosis of thin membrane nephropathy (Fig. 1A). In selected biopsies of diseased kidneys β_6 staining was largely confined to tubular epithelium beyond the ascending loop of Henle (distal convoluted and straight tubules and cortical and medullary collecting ducts). Staining for β_6 in glomeruli and proximal tubules was rarely observed except for occasional patchy staining of the epithelium lining Bowman's capsule in less than 5% of biopsies. There was often a predilection for the juxtaglomerular portion of the distal tubule, including the juxtaglomerular apparatus and adjacent tubular segment and/or cortical collecting duct (Fig. 1B and C). This area was commonly positive in a variety of disease states even when there was little or no staining elsewhere suggesting that it is one of the first areas affected. In some tubular segments the distribution of β_6 appeared to be polarized to basal and lateral cell membranes (Fig. 1D) but in most tubules β_6 staining was seen more diffusely throughout the cell as exemplified in the biopsy of a patient with acute necrotizing glomerulonephritis, (Fig. 1E). There were many examples of biopsies, particularly in allografts with delayed function, in which all the distal tubular cross-sectional elements in the section were positive for β_6 staining (Fig. 1F).

Strong colocalization of expression of β_6 and Tamm-Horsfall protein (a marker for distal tubular epithelia) was observed within tubular cross-sections suggesting that β_6 staining was largely confined to the distal nephron (Fig. 1G and H). Intraluminal protein casts were positive for Tamm-Horsfall protein but not for β_6 (Fig. 1G and H). Intraluminal casts in biopsies of patients with acute tubulitis occasionally could be seen to contain cells staining for β_6 and β_6-stained cuboidal cells, double stained for Tamm-Horsfall protein, were observed in the urine of such patients (Fig. 1I).

In native kidneys with acute renal failure due to tubular necrosis β_6 staining of tubules was diffuse (median score 3+), often despite minimal structural damage obvious on light microscopy (Fig. 1J). This was also the case with renal allografts showing delayed graft function with or without rejection (Fig. 1K and L). In acute renal failure due to severe crescentic glomerulonephritis accompanying interstitial inflammation was associated with very diffuse β_6 staining in tubules (Fig. 1M and N). In severe tubulitis accompanying acute interstitial nephritis or acute severe allograft rejection (Fig. 1O), the inflamed tubules usually expressed β_6. On the other hand, diffuse β_6 expression was also seen when there was obvious chronic structural damage and minimal inflammation in end-stage renal failure kidneys. In these cases β_6 was commonly seen in areas of tubular **a**trophy with interstitial fibrosis (Fig. 1P), and was invariably seen in so-called areas of "thyroidization" where distal tubules were obstructed by protein casts (Fig. 1Q). Myeloma casts were also associated with widespread β_6 expression (Fig. 1R).

Expression of the β_6 integrin subunit in native kidneys

Expression of the epithelial-restricted β_6 integrin subunit was not observed in the tubular epithelium from all seven patients with thin membrane nephropathy confirmed on electron microscopy. In contrast, varying degrees of heterogeneous β_6 staining were defined by immunohistochemistry in paraffin-embedded biopsies obtained from patients suffering from glomerulonephritis (Table 2) (Fig. 2) or miscellaneous kidney diseases (Table 3) (Fig. 3).

Glomerulonephritis. β_6 staining was generally not seen in nonproliferative forms of glomerulonephritis (Table 2)(Fig. 2), with the exception of primary focal and segmental glomerulosclerosis (FSGS) in which mild to moderate focal tubular β_6 staining was seen in 7/7 cases. In biopsies with acute diffuse endothelial or mesangial proliferative glomerulonephritis (postinfectious, IgA nephropathy)β_6 staining was sparse and focal with over

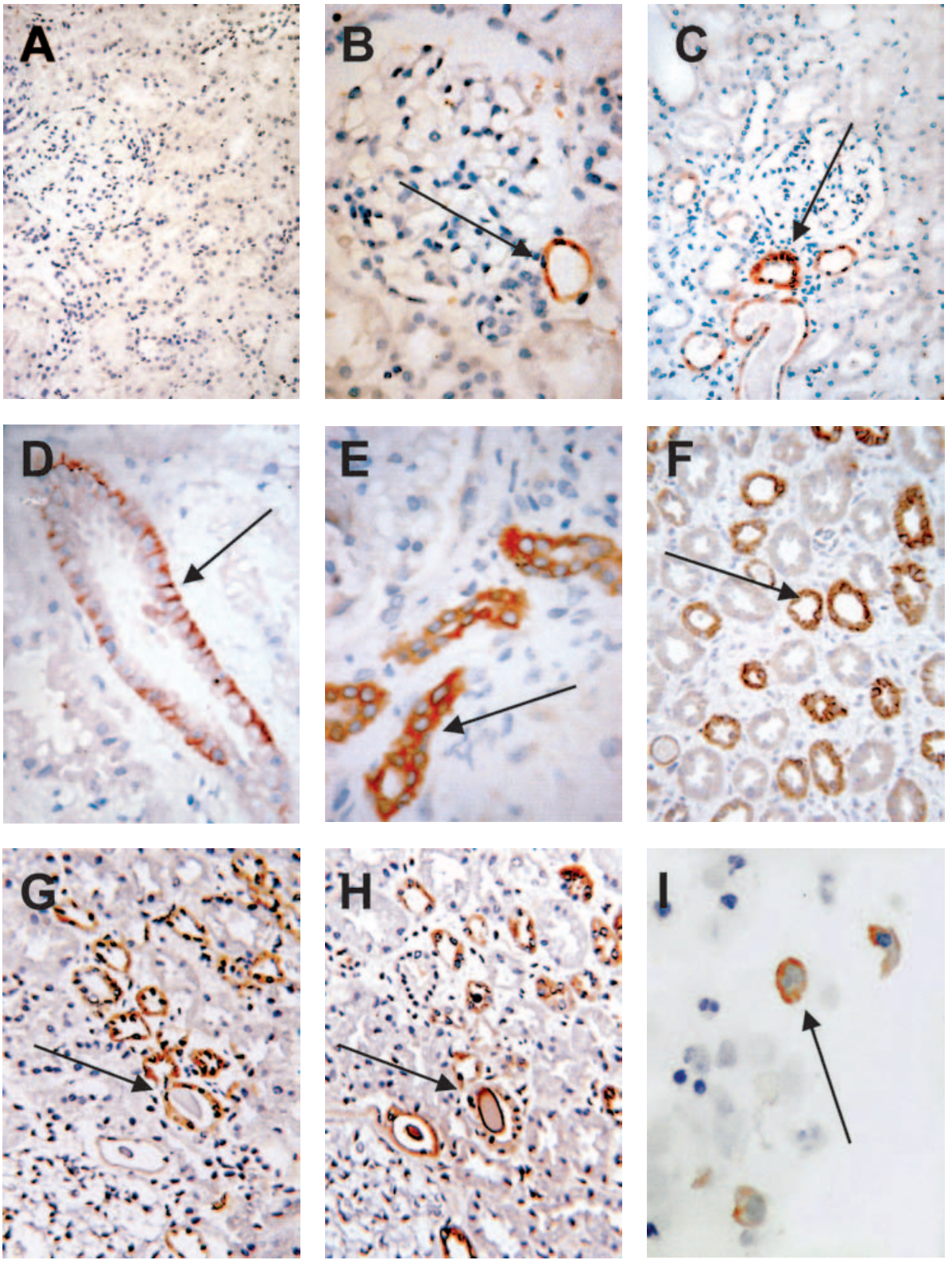

Fig. 1. Typical patterns of immunoperoxidase staining of β_6 integrin in the renal tubular epithelium of native and transplanted kidney biopsies. Photo micrographs of paraffin-embedded sections, prepared and stained for β_6 integrin by the immunoperoxidase technique as described in the text and counterstained with hematoxylin. (*A*) No β_6 expression in the biopsy of a patient with thin membrane nephropathy. (*B*) Isolated staining of cortical collecting duct in region of juxtaglomerular apparatus (arrow) in 3-month protocol biopsy of renal transplant. (*C*) Staining of juxtaglomerular apparatus region (arrow) and spread along adjacent tubule in renal transplant with acute interstitial rejection. (*D*) High power view of single tubule with basal and lateral membrane expression of β_6 (arrow) from allograft with delayed graft function. (*E*) Denser staining throughout the cell

90% of biopsies staining less than 5% of the tubular cross-sections (0 to trace). In contrast, in the more destructive and progressive disease, acute membranoproliferative glomerulonephritis, β_6 staining was present in 100% of biopsies and in 3/7 of these there was diffuse distal tubular staining (score 2 to 3+). Diffuse staining was seen in all cases of focal necrotizing glomerulonephritis with 12/13 having greater than 10% of tubules staining (2 to 4+) compared to 3/20 for the nonnecrotizing proliferative glomerulonephritis cases ($P < 0.001$, Fisher's exact test). Biopsies showing advanced chronic glomerulonephritis (featuring glomerulosclerosis, arteriosclerosis, tubular atrophy, and interstitial fibrosis) whether associated with mesangial IgA deposits or not were also invariably diffusely positive for β_6 (2 to 3+).

Miscellaneous kidney diseases: Tubulointerstitial, metabolic, and thrombotic. Trace to very diffuse staining (trace to 4+) was seen in 14/15 patients with acute (usually drug-induced) interstitial nephritis. For seven patients with acute tubular necrosis all cases exhibited very diffuse β_6 staining (3 to 4+). The biopsy from one patient with ureteric obstruction from retroperitoneal fibrosis was normal to light microscopy but was moderately diffuse (2+) for β_6. In patients suffering from chronic renal failure secondary to chronic interstitial nephritis, β_6 expression was absent in 23% and trace to mildly diffuse (trace to 2+) in the remainder. β_6 staining in this group was less marked than the afore-mentioned chronic glomerulonephritis group (3/13 exhibiting >10% tubular staining compared with 7/7 ($P = 0.002$, Fisher's exact test). In diabetic nephropathy, β_6 staining varied from trace to moderately diffuse (trace to 3+) in 75% of kidneys biopsied. Some of the most diffuse β_6 expression (2 to 4+) was observed in thrombotic microangiopathy (HUS/TTP) involving the majority of nephrons in 10/10 patients studied. Similarly, very diffuse staining for β_6 (4+) was seen in 2/2 patients with acute renal failure due to myeloma kidney (Table 3) (Fig. 3).

Expression of the β_6 integrin subunit in renal transplants

The data for time zero and 3-month "protocol" biopsies from 50 sequential transplant recipients are presented in Table 4 and Figure 4. Selected "service" biopsies performed for allograft dysfunction in 40 transplant recipients are presented in Table 5 and Figure 5.

Biopsies performed routinely: "Protocol" biopsies

On table, postreperfusion biopsies (time zero). Despite the traumatic circumstances surrounding the death of many kidney donors, the vast majority (98%) of time zero renal biopsies revealed no up-regulation of β_6 in their tubules as indicated in Table 4 and Figure 4. The one case of 3+ staining had no particular distinguishing histological or clinical features.

Three-month "protocol" biopsies. At 3 months post-transplant, 33/50 (66%) of biopsies revealed some β_6 staining. Fourteen biopsies (28%) demonstrated subclinical acute rejection (SAR) with the following Banff '97 scores: suspicious, 10; 1a, 3; and 1b, 1. Eleven of 14 (79%) of these showed β_6 positivity to varying degrees (trace to 2+). Twenty-six biopsies (52%) showed evidence of CAN with Banff scores: 1a, 9; 1b, 15; and 2b, 2. Of these, 21/26 (81%) showed β_6 positivity (trace to 4+). In seven cases CAN and SAR coexisted with only one such biopsy being β_6 negative. However, β_6 positivity (trace to 2+) was also observed in 8/17 biopsies (47%) with minor transplant changes only.

Biopsies performed for graft dysfunction: "Service" biopsies

All 40 biopsies were positive for β_6 to varying degrees as listed in the following subcategories.

DGF. Nine biopsies were from recipients whose grafts failed to function postoperatively and were found to have acute tubular necrosis, predominantly as a result of prolonged cold ischemia, and not complicated by acute rejection. As shown in Table 5 and Figure 5, β_6 staining was moderate to very diffuse in 8/9 cases (3 to 4+) and mildly diffuse (2+) in the remaining patient. The earliest biopsy was performed on day 2 and already showed very diffuse staining for β_6.

DGF + ARE. In a further three cases of initial nonfunction there was acute interstitial rejection as well as

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of distal tubule (arrow) in a patient with acute necrotizing glomerulonephritis. (*F*) Widespread staining of distal tubules (arrow) with sparing of proximal tubules in allograft with delayed graft function. (*G*) Group of distal tubules stained for β_6 [note sparing of intraluminal cast (arrow)]. (*H*) Same group of tubules from adjacent section showing concordant staining of tubules and casts (arrow) for Tamm-Horsfall protein, a marker for distal tubules and collecting ducts. (*I*) Dual staining for β_6 (brown) and Tamm-Horsfall protein (red) of cells (arrow) in the urinary sediment of a patient with acute interstitial nephritis [magnification (A) ×100; (B, C, F, G, and H) ×250; (D, E, and I) ×400, plus up to ×3 camera optical zoom]. (*J*) Low power view of very diffuse (4+) β_6 staining of distal tubules in native kidney with acute tubular necrosis. (*K*) Similar staining in day 5 biopsy of renal transplant with delayed graft function. (*L*) Similar very diffuse staining of tubules cut longitudinally from renal transplant suffering delayed graft function and acute rejection. (*M*) Low power view of patient with crescentic glomerulonephritis. (*N*) High power view of framed area in previous section showing only distal tubular staining for β_6 with sparing of glomerular epithelium and crescent. (*O*) Distal tubulitis in a patient with acute interstitial rejection showing heavy β_6 staining of the invaded epithelium (arrow). (*P*) Virtually all remaining tubules (arrow) stain for β_6 in a patient with severe end-stage chronic allograft nephropathy. (*Q*) β_6 staining of flattened epithelium (arrow) of dilated tubule containing hyaline cast ("thyroidization") in patient with chronic interstitial nephritis. (*R*) β_6 staining of tubules containing fractured casts (arrow) in patient with multiple myeloma [magnification (J and M) ×100; (K, L, N, P, Q, and R) ×250; (O) ×400, plus up to ×3 camera optical zoom].

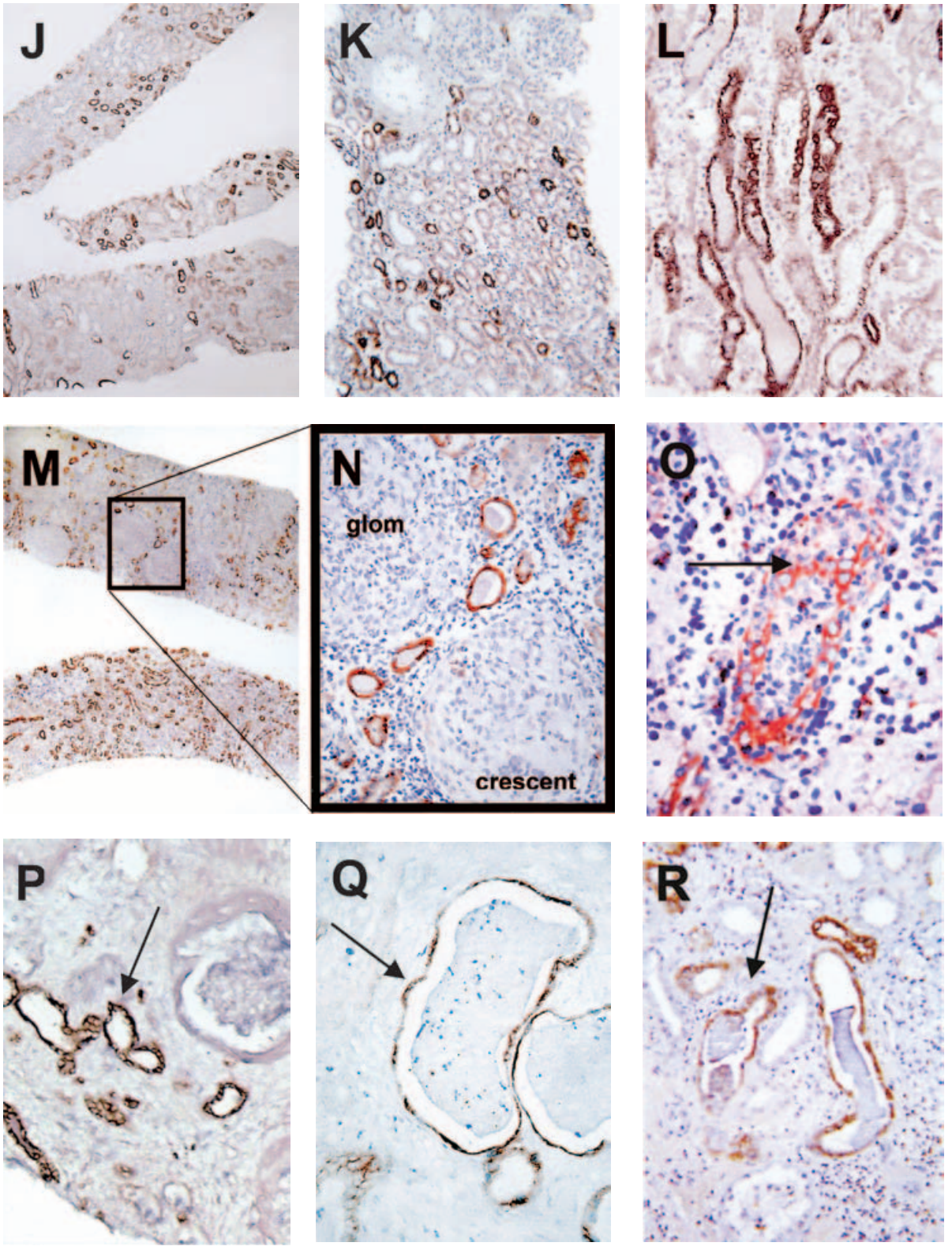

Fig. 1. continued

Table 2. β_6 integrin expression in glomerulonephritis

Renal disease category	Histologic diagnosis	No.	% with β_6 staining	Number of biopsies in each β_6 score range					
				0	Trace	1+	2+	3+	4+
"Normal"	Thin membrane nephropathy	7	0	7					
Non proliferative	Minimal lesion nephrotic syndrome;	7	0	7	—	—	—	—	—
glomerulonephritis	membranous nephropathy;	5	0	5	—	—	—	—	—
	primary focal segmental glomerulosclerosis	7	100	—	2	2	2	1	—
Acute	Diffuse Endocapillary glomerulonephritis;	3	67	1	1	1	—	—	—
proliferative	mesangial IgA disease;	10	70	3	7	—	—	—	—
glomerulonephritis	membranoproliferative glomerulonephritis	7	100	—	2	2	2	1	
Focal	Henoch-Schonlein vasculitis;	2	100	—	—	—	—	2	—
necrotizing	pauci-immune vasculitis;	8	100	—	—	1	1	3	3
glomerulonephritis	anti glomerular basement membrane disease	3	100	—	—	—	—	—	3
Chronic	Chronic mesangial IgA disease;	4	100	—	—	—	2	2	—
glomerulonephritis	end-stage glomerulonephritis (unknown)	3	100	—	—	—	1	2	—

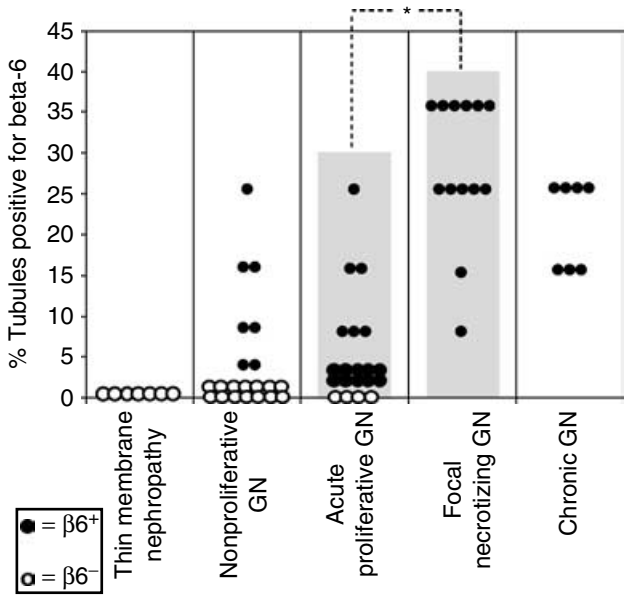

Fig. 2. Distribution of averaged β6 scores as assessed by immunohistochemistry in paraffin-imbedded biopsies obtained from patients suffering from glomerulonephritis. *Denotes statistically significant difference in the proportion of biopsies with greater than 10% tubular staining for β6 between acute proliferative versus focal necrotizing glomerulonephritis ($P < 0.001$; Fisher exact test).

acute tubular necrosis on biopsy and these were all diffusely positive (3 to 4+) for β_6.

AREs. In 11 recipients considered to have clinical reversible AREs (Banff scores 1a to 2b), all biopsies were positive for β_6 (1 to 4+).

CAN. All 18 recipients in this group were biopsied because of chronic deterioration in graft function. All biopsies were positive for β_6 (trace to 4+).

DISCUSSION

We report herein the first description, in any tissue, of an immunostaining technique for the β_6 subunit applicable to formalin-fixed paraffin sections. This has allowed us to study β_6 expression in archival biopsies of patients chosen to reflect virtually the entire spectrum of renal diseases in native kidneys and renal transplants. This study provides indirect evidence that this integrin is likely to be important in the genesis of tubular injury and repair. By associating patterns of β_6 expression with known disease mechanisms we can speculate about the types of injury that may lead to up-regulation of this cell surface molecule and why its persistence may be a marker of disease progression.

Overall, 64% (170/267) of biopsies selected for this study were positive for β_6 staining to a varying degree. In many ways the biopsies ($N = 97$) that did not express β_6 were as informative as those that did. In biopsies from native kidneys, β_6 was not expressed by the tubular epithelium in thin membrane nephropathy, a surrogate for normal kidneys. Likewise, β_6 was not expressed in nonproliferative glomerular diseases associated with the nephrotic syndrome with the notable exception of FSGS. It tends to be expressed focally or not at all in nonnecrotizing proliferative glomerular disease such as mesangial IgA disease or poststreptococcal glomerulonephritis (although it is clearly more prominent in membranoproliferative glomerulonephritis). However, it is seen in all cases of focal necrotizing glomerulonephritis with significantly more diffuse staining than in the nonnecrotizing group. The difference may be that in the necrotizing group there is commonly an interstitial component to the inflammatory response and tubular ischemia secondary to occlusion of glomerular capillary tufts. In this regard it is instructive that very high levels of β_6 expression are seen, by both Breuss and us, in primary HUS where glomerular thrombosis severely impairs tubular hemoperfusion but without an obvious interstitial inflammatory infiltrate.

In diseases characterized by acute or chronic tubulointerstitial injury/inflammation strong up-regulation of β_6 is observed. Acute tubular necrosis induced by

Table 3. β_6 integrin expression in miscellaneous causes of kidney failure

Renal disease category	Histologic diagnosis	No.	% with β_6 staining	Number of biopsies in each β_6 score range					
				0	Trace	1+	2+	3+	4+
Acute renal failure	Acute interstitial nephritis;	15	93	1	2	3	3	4	2
	acute tubular necrosis;	7	100	—	—	—	2	2	3
	obstructive uropathy	1	100	—	—	—	1	—	—
Chronic renal failure	Chronic interstitial nephritis	13	77	3	4	3	3	—	—
Metabolic	Diabetic nephropathy;	12	75	3	3	3	1	2	-
	myeloma kidney	2	100	—	—	—	—	—	2
Thrombotic	HUS/TTP	10	100	—	—	—	2	4	4

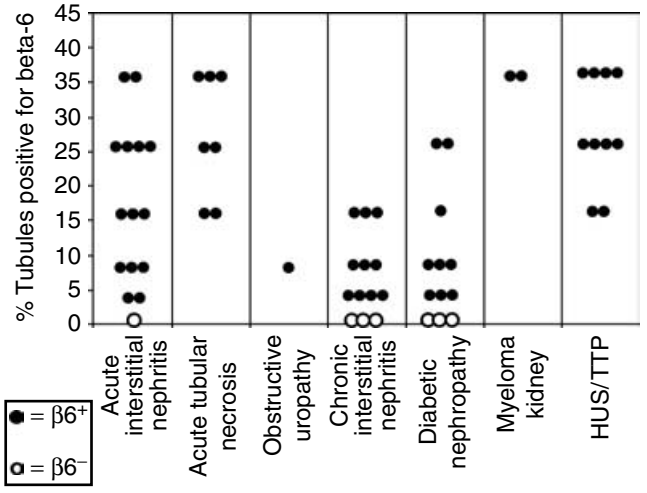

Fig. 3. Distribution of averaged β6 scores as assessed by immunohistochemistry in paraffin-imbedded biopsies obtained from patients suffering from miscellaneous causes of renal failure.

hypoperfusion of native kidneys is associated with diffuse β_6 expression. It is also invariably expressed when the tubulointerstitium is the primary inflammatory target such as in acute interstitial nephritis of varying etiologies (toxic, septic, and immune-mediated). The very prominent β_6 staining in the two cases of multiple myeloma suggests that this integrin may also be up-regulated in response to intraluminal irritants. In one patient with pelvicalyceal dilatation from incomplete ureteric obstruction due to retroperitoneal fibrosis the expression of moderate amounts of β_6 may indicate a response to pyelotubular backpressure. In chronic renal diseases such as chronic glomerulonephritis, diabetic glomerulosclerosis, and analgesic nephropathy, the surviving tubules in areas of tubular atrophy and replacement fibrosis usually express β_6, often very diffusely. However, chronic glomerular disease was associated with significantly more β_6 expression than chronic interstitial nephritis. This finding may support the hypothesis generated from the HUS biopsies that obliterative glomerular disease reducing tubular hemoperfusion is a potent trigger to β_6 up-regulation in distal tubules.

In the present study we also examined a series of "service" and "protocol" biopsies from renal transplant recipients. Index biopsies were chosen from patients with DGF (with or without acute rejection), AREs, and from patients suffering from chronic graft deterioration. Our findings from the "on the table" protocol biopsies indicate that the β_6 integrin subunit is not usually found in adult kidneys harvested for renal transplantation (1/50). This almost complete negative finding was unexpected given the cytokine release known to accompany brain death and the likelihood that some donors would have suffered periods of renal hypoperfusion. However, it is further evidence that normal kidneys do not express β_6 and that it takes some time for up-regulation of β_6 after ischemia/reperfusion. By 3 months β_6 is expressed in varying degrees in two thirds of transplant protocol biopsies. At that time point it was more commonly expressed in patients with subclinical rejection (79%) and with early CAN (81%) than in patients with "normal" biopsies (47%).

The selected "service" biopsies, performed for graft dysfunction, show that β_6 was up-regulated in 100% of cases. DGF was strongly associated with β_6 expression with 92% having moderate to very diffuse staining (more than 20% of tubular cross-sections). The earliest biopsy in this group was on day 2 posttransplant and already had 3+ staining despite a negative time zero biopsy. It is not yet known if β_6 expression occurs in cadaveric kidneys that function immediately as early protocol biopsies were not performed in these patients. Grafts with good primary function who subsequently reject also show diffuse β_6 but possibly of a lesser grade than the DGF kidneys. All the grafts in the CAN group were biopsied for severe and progressive graft dysfunction and all showed β_6 staining with 61% being diffuse. In many of these biopsies, the presence of quite marked tubular atrophy meant that often most of the remaining tubular cross-sections consisted of dilated distal tubules expressing β_6.

The rapidity with which this integrin is up-regulated in allografts that fail to function suggests that ischemia/reperfusion is a major stimulus to its expression by tubular epithelium. It is known that integrins are involved in the inflammatory response to ischemia/

Table 4. β_6 integrin expression in protocol biopsies

Renal treatment disease category	Histologic diagnosis	No.	% with β_6 staining	Number of biopsies in each β_6 score range					
				0	Trace	1+	2+	3+	4+
On table, T_0	Mild perfusion changes	50	1(2%)	49	—	—	—	1	—
3-month	Minor transplant changes;	17	8(44%)	10	3	3	1	—	—
protocol	subclinical acute rejection (SAR)[a]	14	11(79%)	3	3	5	3	—	—
biopsies	chronic allograft nephropathy (CAN)[a]	26	21(81%)	5	8	9	3	—	1[b]

[a]Seven patients had CAN and SAR and are represented in both groups.
[b]Graft was obstructed by lymphocele.

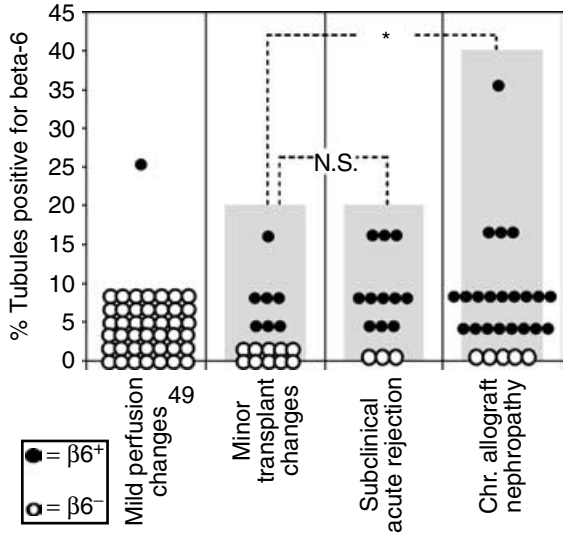

Fig. 4. Distribution of averaged β6 scores as assessed by immunohistochemistry in paraffin-imbedded tissue obtained from "protocol" biopsies in renal transplant recipients. *Denotes statistically significant difference in the proportion of biopsies exhibiting any β6 tubular staining in chronic allograft nephropathy versus minor transplant changes ($P = 0.01$; Fisher exact test). N.S. denotes no statistically significant differences in proportion of biopsies exhibiting any β6 tubular staining in patients with SAR versus minor transplant changes ($P = 0.07$; Fisher exact test).

reperfusion injury. Ischemia/reperfusion injury involves adhesion of leukocytes to the activated endothelium leading to tissue damage. Therapeutic strategies that target leukocytes using either monoclonal antibodies, antisense oligonucleotides, or gene knockout blockade of β_2 integrins and intercellular adhesion molecule-1 (ICAM-1) have been shown to attenuate acute renal failure in experimental models of renal ischemia [15]. Mononuclear cell infiltrates are found in ischemia/reperfusion injury and it has been suggested that adhesion of infiltrating T cells to renal tubular cells may provide a potential mechanism underlying postischemia tubular dysfunction [16]. However, it remains to be determined whether the main stimulus to β_6 is hypoxia alone or the known inflammatory response that accompanies reperfusion, or both.

In acute rejection most lymphocytic tubulitis involves the distal tubule and collecting ducts [17, 18], which is the common site for β_6 expression, raising speculation that β_6 may partake in the alloimmune response. β_6 expression does colocate with areas of active tubulitis although not invariably suggesting that, in acute rejection, mechanisms other than the interaction between tubule and cytotoxic T cell may be important in the de novo expression of β_6. Later, the expression of β_6 in chronically failing grafts could be secondary to (chronic) ischemia/hypoxia brought about by thickening of basement membranes of peritubular capillaries but could also be the consequence of direct tubular injury from chronic inflammatory cells or drug toxicity.

The use of counterstained paraffin-imbedded sections enabled us to determine that β_6 staining was restricted nearly exclusively to the distal tubules and collecting ducts. In double staining experiments β_6 was closely colocated with Tamm-Horsfall protein, a marker of distal tubules [19]. Distal tubular cells are the site of constitutive expression of a number of regulatory cytokines such as osteopontin [20], regulated upon activation, normal T cell expressed and secreted (RANTES) [21], and monocyte chemoattractant protein-1 (MCP-1) [22] suggesting that distal tubular epithelial elements may be more active than proximal segments in maintaining the integrity of the tubulointerstitium at times of injury. Following ischemia/reperfusion injury necrosis has been shown to occur mainly in the proximal tubules, while the distal nephron survives [23]. The mechanism(s) whereby distal tubule cells remain relatively protected from ischemia appears to involve the mitogen-activated protein (MAP) kinase signaling pathway. For example, in an animal model, activation of the MAP kinases called extracellular signal-regulated kinases (ERKs) following oxidant injury has been observed only in the ascending limb but not proximal tubule cells [23]. We have recently reported that the cytoplasmic domain of the β_6 integrin subunit binds directly to ERK2 [24]. Whether β_6-bound ERK2 within the distal renal tubule cells plays a role in protection from ischemic injury remains to be investigated but it does raise the possibility that β_6 up-regulation in the distal nephron may act as a survival signal. In chronic renal disorders, the inflammatory cytokine transforming growth factor beta (TGF-β) is thought to play a major role in promoting tubulo-epithelial hypertrophy and fibrosis [25]. The $\alpha_v\beta_6$ integrin binds not only matrix ligands such fibronectin

Table 5. β_6 integrin expression in service biopsies

Renal treatment disease category	Histologic diagnosis	No.	% with β_6 staining	Number of biopsies in each β_6 score range					
				0	Trace	1+	2+	3+	4+
Graft dysfunction	Delayed graft function	9	9(100%)	—	—	—	1	5	3
	DGF + Acute rejection	3	3(100%)	—	—	—	—	1	2
	Acute rejection	11	11(100%)	—	—	6	1	3	1
	Chronic allograft nephropathy	18	18(100%)	—	1	6	2	6	3

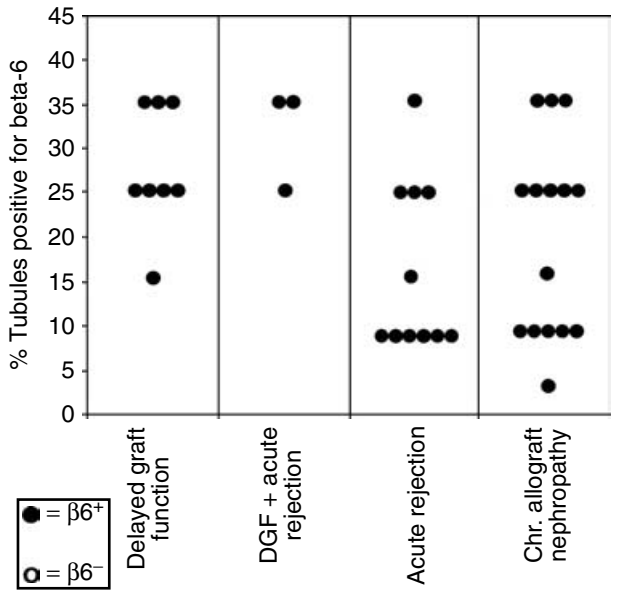

Fig. 5. Distribution of averaged β6 scores as assessed by immunohistochemistry in paraffin-imbedded tissue obtained from "service" biopsies of dysfunctional renal allografts.

and tenascin but has also been shown to bind and activate latent TGF-β in normal and malignant cells [26]. TGF-β can itself up-regulate β_6 expression in epithelial cells [27, 28] raising the possibility that TGF-β autoregulation of β_6 expression occurs in tubular cells of chronically hypoperfused kidneys.

Following renal transplantation, a strong link has been observed between the occurrence of AREs and the eventual development of chronic rejection leading to graft loss. This has lead to the search for an early marker that will identify those grafts at risk of chronic rejection and, thereby, allow for early use of rescue strategies. Despite an exhaustive examination of different proteins, cytokines, and inflammatory cells in early posttransplant biopsies, only a few markers have proved to have value in predicting progressive graft failure in a cohort of apparently stable grafts [29]. None of these is a specific marker of ongoing damage to the tubular epithelium. In our study it appears that de novo β_6 expression in renal transplants results predominantly from acute tubular ischemia/reperfusion injury at the time of transplantation. De novo expression may also occur from direct interaction of immune effector cells with distal tubular epithelium as witnessed by the ten patients with no clinically obvious DGF preceding their acute rejection (although early protocol biopsies would be necessary to exclude prior expression of β_6 in these patients). Subsequently, reversible and/or irreversible vascular changes secondary to rejection or drug nephrotoxicity may further add to the ischemic damage and thereby enhance β_6 expression. It seems likely that β_6 is up-regulated each time the allograft suffers one of these "hits" and its presence in protocol biopsies may be a useful marker to identify patients with a long-term unfavorable outcome following transplantation. Studies to address this are ongoing in our unit.

The reason for the negative findings in a few biopsies expected to be positive is unclear but may relate to lack of success in antigen retrieval and hence antibody binding. Alternatively, there may be some individuals who fail to express β_6 when provoked. The study is retrospective and the biopsies were selected arbitrarily from disease categories without quantifying duration or severity. Only in the case of the protocol transplant biopsies does it reflect consecutive patient sampling of a single cohort. Like other studies we are assuming that thin membrane nephropathy equates with normal controls. We did not set out to look at clinical correlations of β_6 staining or its time course in iterative biopsies. However, this study is the first comprehensive description of patterns of $\alpha_v\beta_6$ expression in renal disease and transplantation. From it we conclude that $\alpha_v\beta_6$ integrin is likely to be a pivotal molecule in the genesis of renal injury and repair and warrants further study at a clinical and molecular level.

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